SUPPLEMENTAL FIGURES

FIGURE S1, related to Figure 1

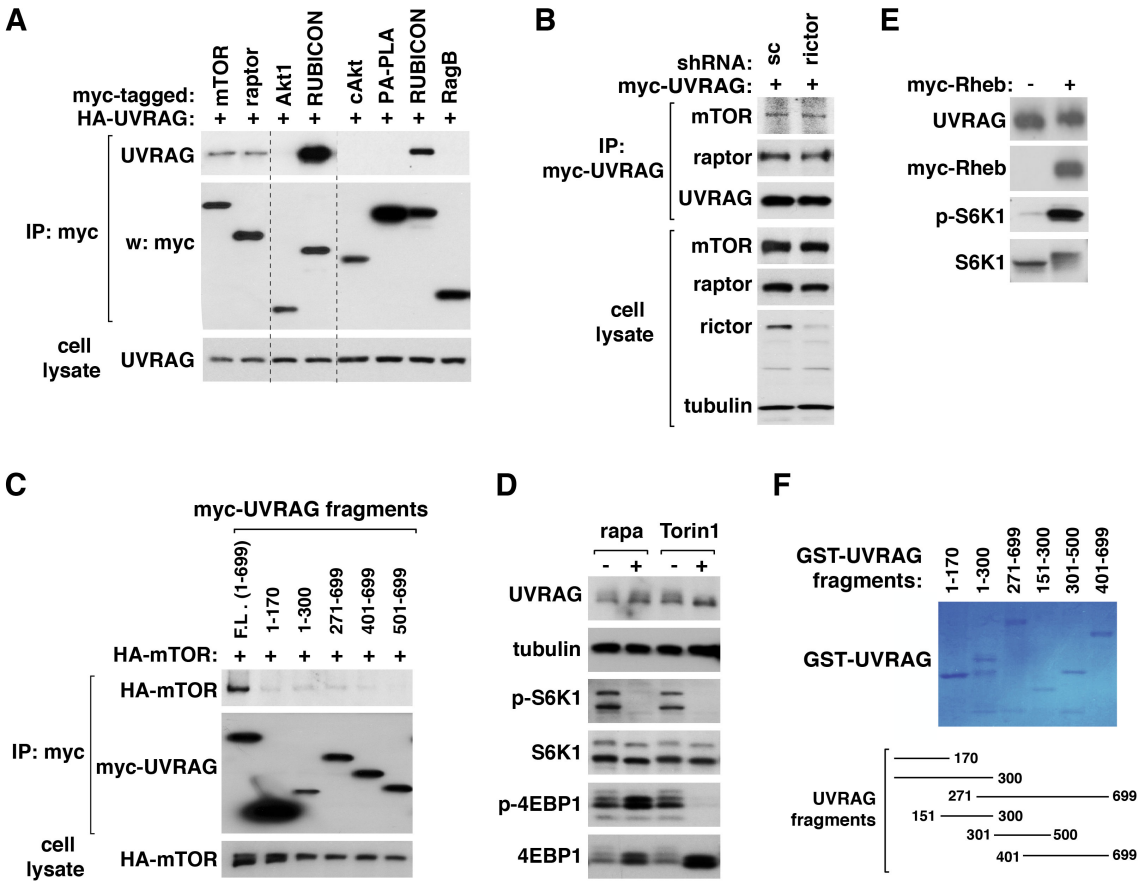


Figure S1. mTOR interacts with UVRAG and induces a mobility shift of UVRAG on SDS-PAGE. (**A**) mTOR and raptor bind to UVRAG. HEK293T cells were transiently transduced with plasmids encoding the indicated genes. Two-days post-transfection, myc immunoprecipitates were analyzed for the interaction. Irrelevant lanes in the middle were removed. (**B**) Rictor knockdown does not affect the interaction between UVRAG and mTORC1. (**C**) The full-length of UVRAG is required for stable binding to mTOR. Myc-tagged full-length and fragments of UVRAG were expressed with HA-mTOR in HEK293T cells where endogenous UVRAG was silenced. The amount of HA-mTOR co-immunoprecipitated with the UVRAG constructs was analyzed by WB. (**D**) Rapamycin does not alter the mobility of UVRAG band on SDS-PAGE. HEK293T cells were treated with either rapamycin (rapa; 100 nM) or Torin1 (250 nM) for 1 h. As vehicle (-), DMSO was used in the same volume. The mobility of UVRAG band on SDS-PAGE as well as the phosphorylation states of S6K1 (Thr389) and 4EBP1 (Thr37/46) were analyzed by WB. (**E**) Rheb overexpression induces a mobility shift of UVRAG. Myc-Rheb was transiently overexpressed in HEK293T cells. Cell lysate was analyzed by WB. (**F**) UVRAG fragments purified by use of a bacterial expression system. Proteins were stained with Coomassie blue G-250.

FIGURE S2, related to Figure 2

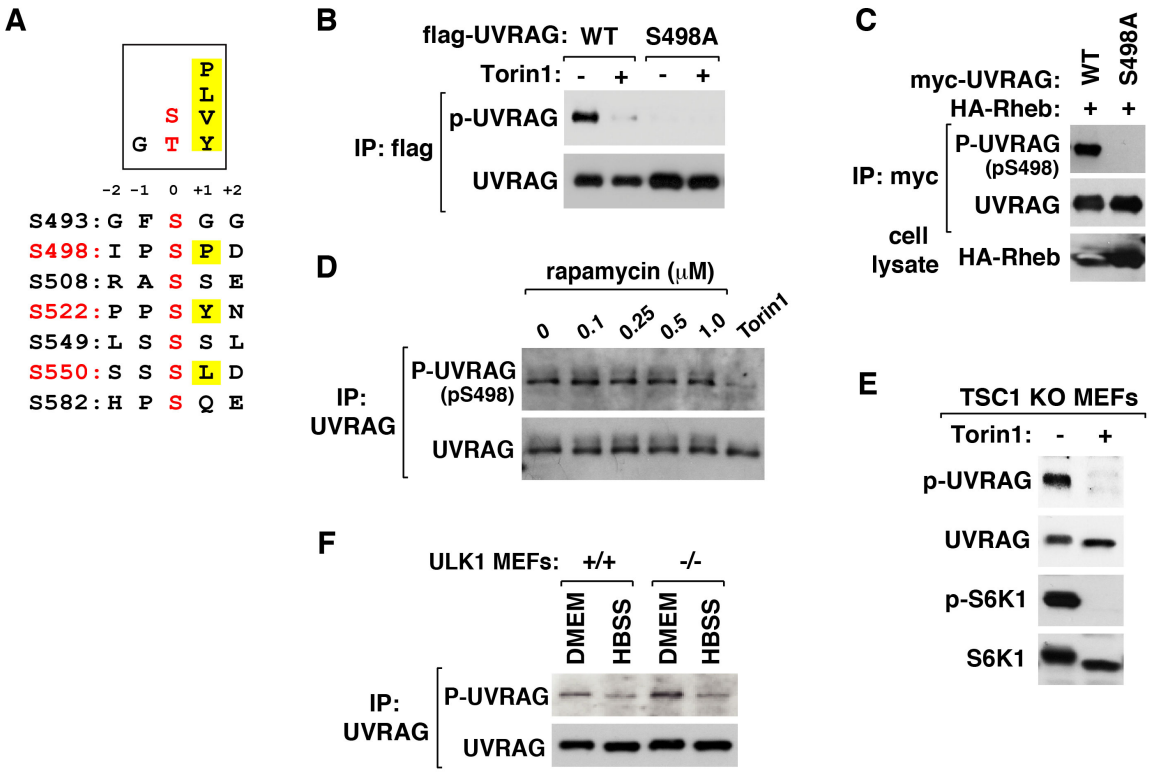


Figure S2. UVRAG Ser498 is an mTORC1-dependent phosphorylation site.
(**A**) Sequence alignments of the residues surrounding the phosphorylation sites of UVRAG identified by mass spectrometry. The serine residues in red show a common feature of the known mTORC1 substrates (Kang et al., 2013). (**B**) Validation of the polyclonal antibody specific to Ser498 phosphorylation. Flag-tagged UVRAG was isolated by immunoprecipitation using anti-flag antibody-conjugated agarose. The phosphorylation state of UVRAG Ser498 was analyzed by WB using the phospho-specific antibody. The polyclonal antibody detected UVRAG WT but not S498A mutant. Treatment of HEK293T cells with Torin1 (250 nM) for 1 h drastically suppressed the detection of the UVRAG band. (**C**) mTORC1 activation specifically induces Ser498 phosphorylation. HA-tagged Rheb was expressed together with myc-tagged UVRAG WT or S498A in HEK293T cells, where endogenous UVRAG was silenced. Myc immunoprecipitates were analyzed by WB with use of the polyclonal antibody. (**D**) Rapamycin does not inhibit the Ser498 phosphorylation. HEK293T cells were treated with rapamycin (0, 0.1, 0.25, 0.5, or 1 μM) or Torin1 (250 nM) for 1 h. The phosphorylation of UVRAG Ser498 was analyzed by WB. (**E**) The polyclonal antibody detected the phosphorylation of endogenous UVRAG in cell lysate. TSC1 KO MEFs were treated with Torin1 as described in (B). Cell lysate was obtained, and the phosphorylation state of UVRAG and S6K1 (pT389) were analyzed by WB. (**F**) ULK1 is not required for the UVRAG phosphorylation. ULK1 MEFs were cultured in Dulbecco's Modified Eagle's medium (DMEM) or Hank's Buffered Salt Solution (HBSS) for 2 h. The phosphorylation of Ser498 was analyzed as described in (B).

FIGURE S3, related to Figure 3

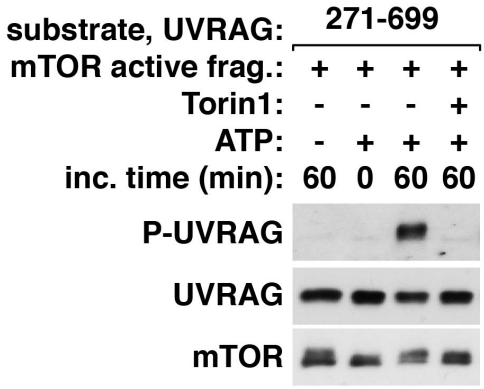


Figure S3. mTOR phosphorylates UVRAG at Ser498. The active fragment of mTOR containing residues 1362-2549 was incubated in the presence or absence of ATP with the UVRAG 271-699 fragment purified from a bacterial expression system. Torin1 (250 nM) was added during the reaction, and the phosphorylation state of UVRAG Ser498 was analyzed by WB.

FIGURE S4, related to Figure 4

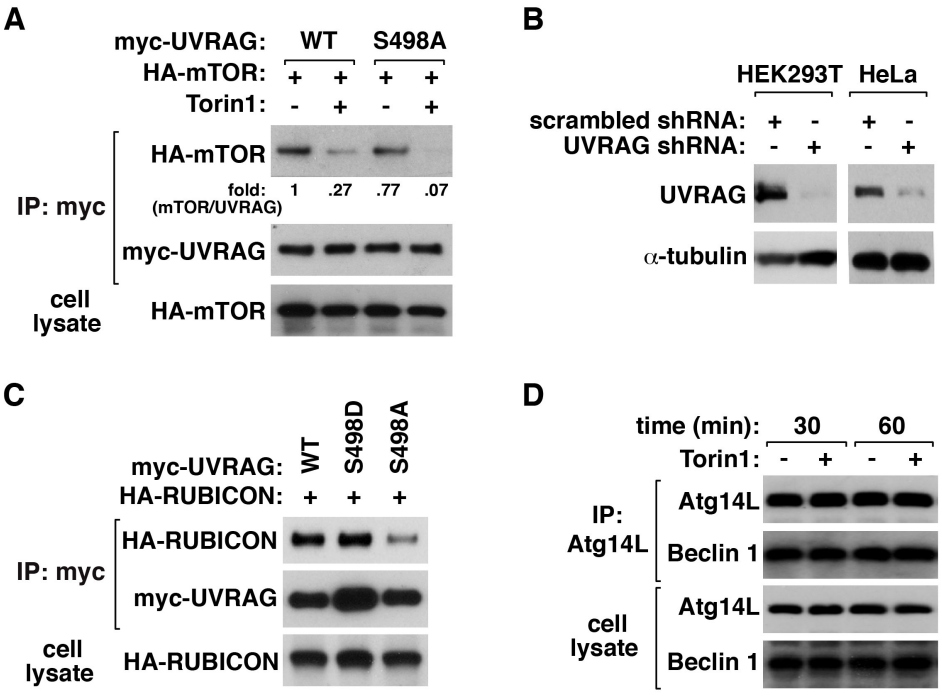
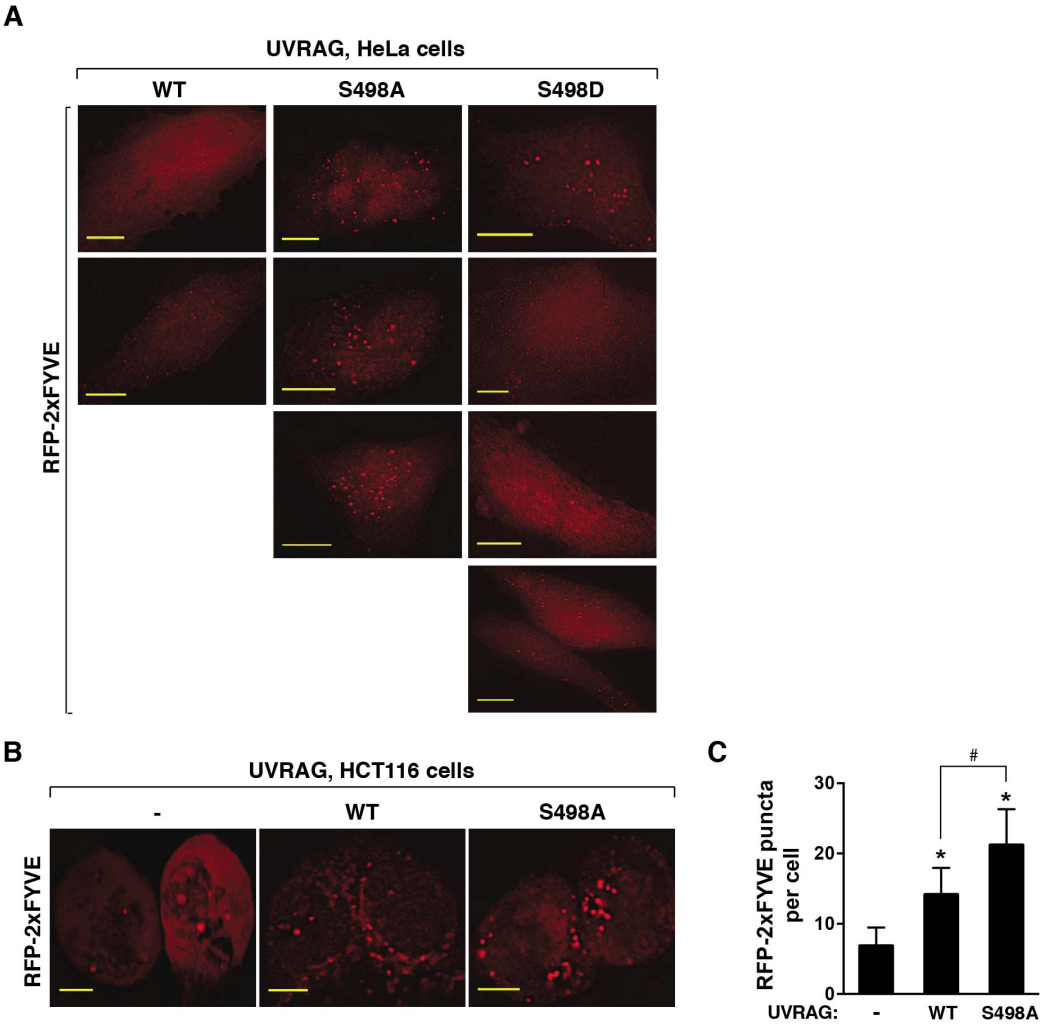
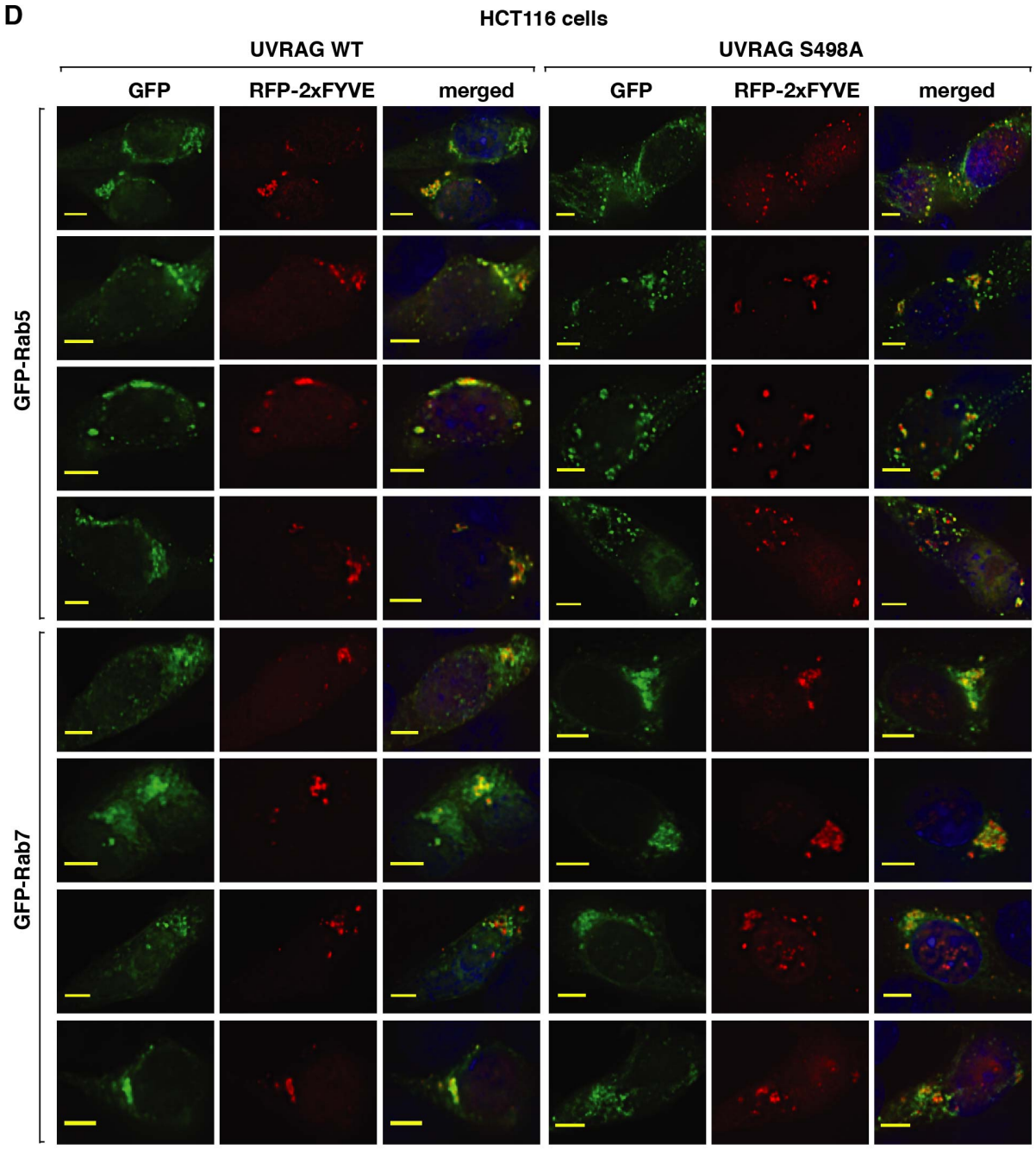


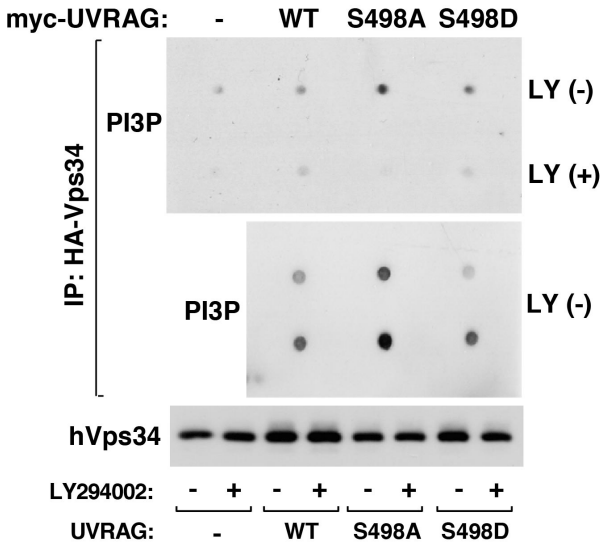
Figure S4. UVRAG S498A mutation reduces the interaction between UVRAG and RUBICON. (**A**) Ser498 phosphorylation state is not critical for the regulation of the UVRAG-mTORC1 interaction. Myc-UVRAG WT or S498A was transiently expressed with HA-mTOR in endogenous UVRAG-depleted HEK293T cells. The transfected cells were treated with Torin1 for 1 h. The amount of HA-mTOR bound to myc-UVRAG was analyzed by WB. (**B**) Knockdown of UVRAG in HEK293T and HeLa cells. The knockdown was achieved by lentivirally transducing the cells with shRNA targeting a 3'-UTR sequence of UVRAG mRNA (see details in Experimental Procedures). (**C**) Ser498 phosphorylation state regulates the interaction between UVRAG and RUBICON. HA-tagged RUBICON was co-expressed with myc-tagged UVRAG WT or mutant constructs in HEK293T cells, where endogenous UVRAG was silenced. The amount of HA-RUBICON recovered with myc-UVRAG immunoprecipitates was analyzed by WB. (**D**) Torin1 does not alter the binding affinity of the interaction between Beclin 1 and Atg14L. HEK293T cells were treated with vehicle (DMSO) or Torin1 (250 nM) for the indicated period of time. The amounts of Atg14L and Beclin 1 recovered with Atg14L immunoprecipitates were analyzed by WB.

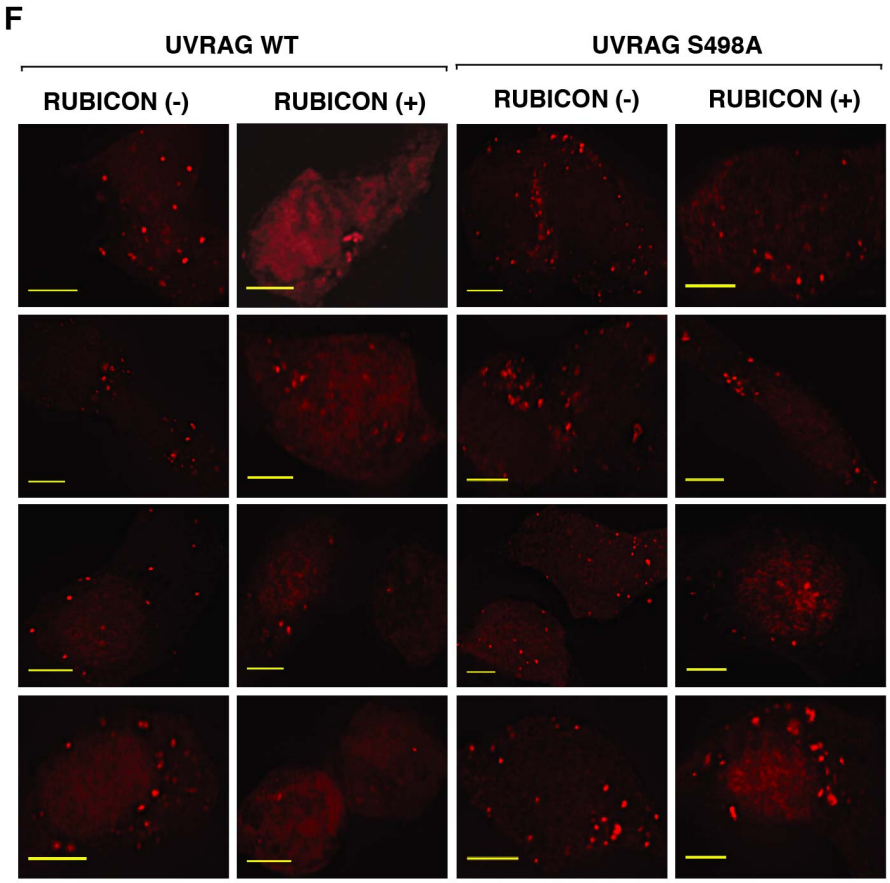
FIGURE S5, related to Figure 5

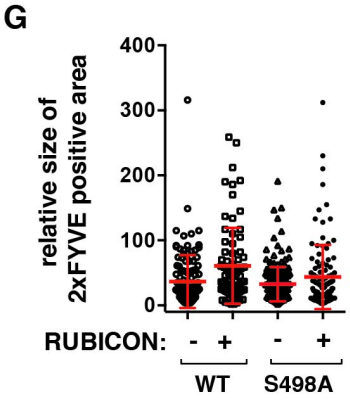



D
HCT116 cells
UVRAG WT
UVRAG S498A
GFP
RFP-2xFYVE
merged
GFP
RFP-2xFYVE
merged
GFP-Rab5
GFP-Rab7

E


myc-UVRAG: - WT S498A S498D
IP: HA-Vps34
PI3P
LY (-)
LY (+)
PI3P
LY (-)
hVps34
LY294002: - + - + - + - +
UVRAG: - WT S498A S498D


F
UVRAG WT
UVRAG S498A
RUBICON (-)
RUBICON (+)
RUBICON (-)
RUBICON (+)


G
relative size of
2xFYVE positive area
400
300
200
100
0
RUBICON: - + - +
WT
S498A

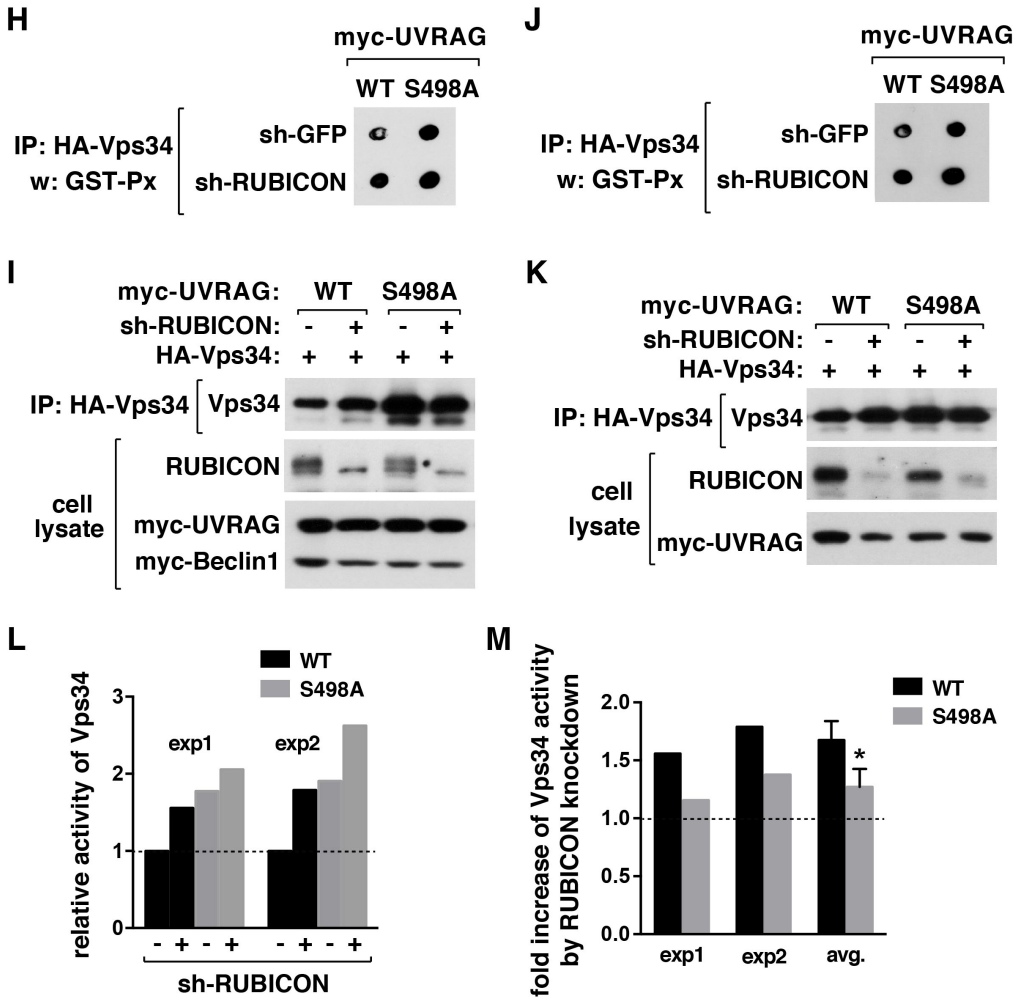
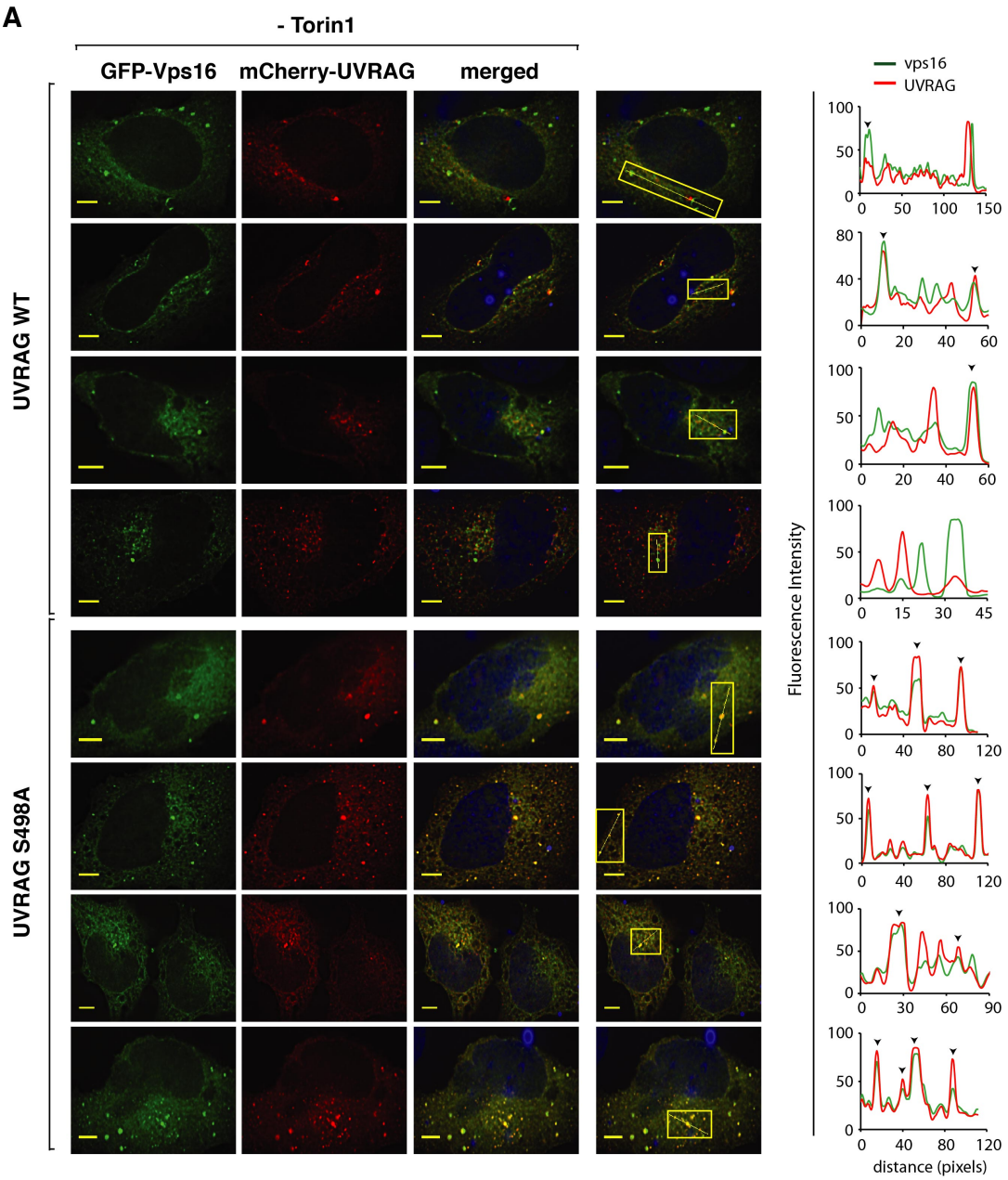
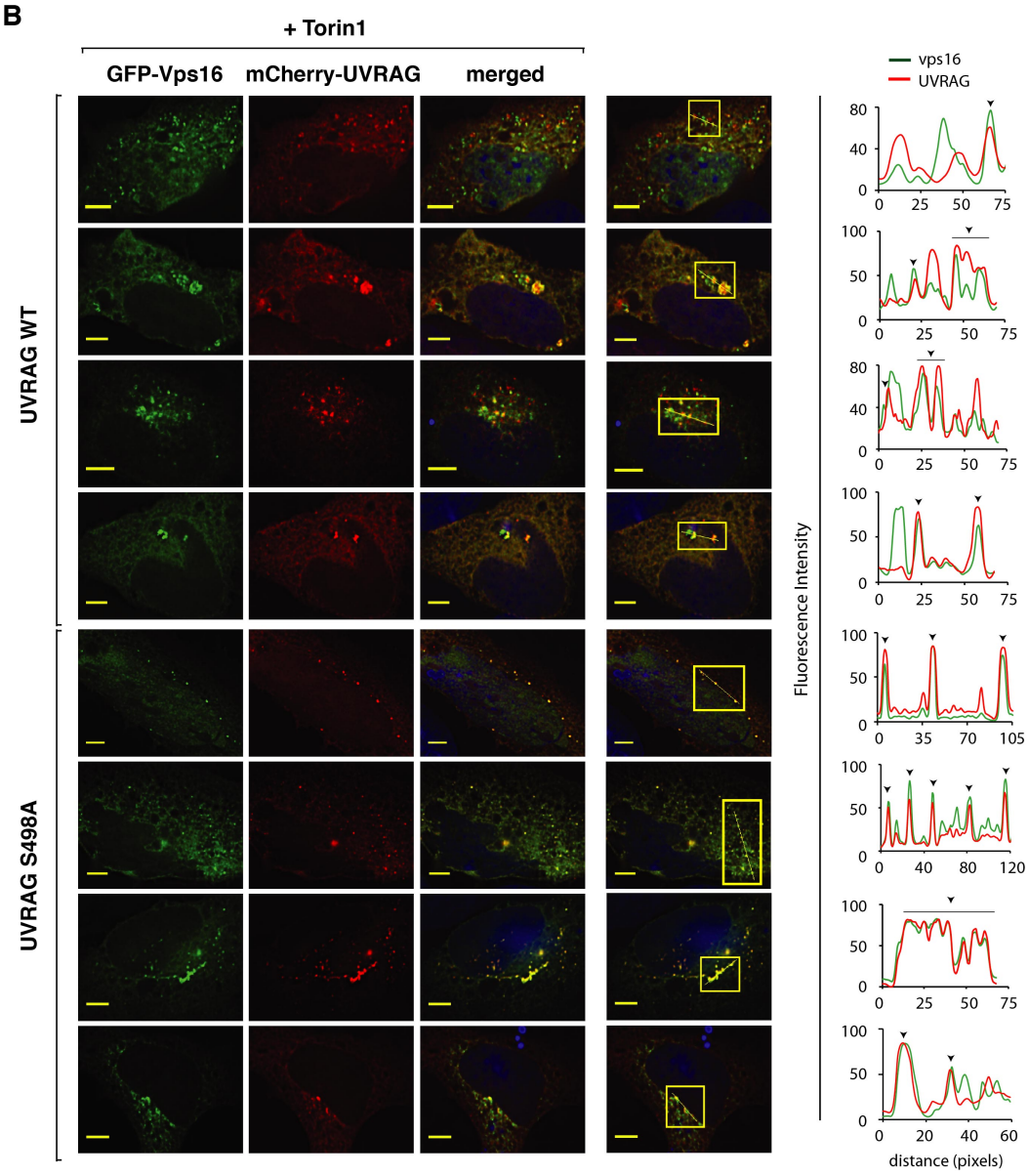
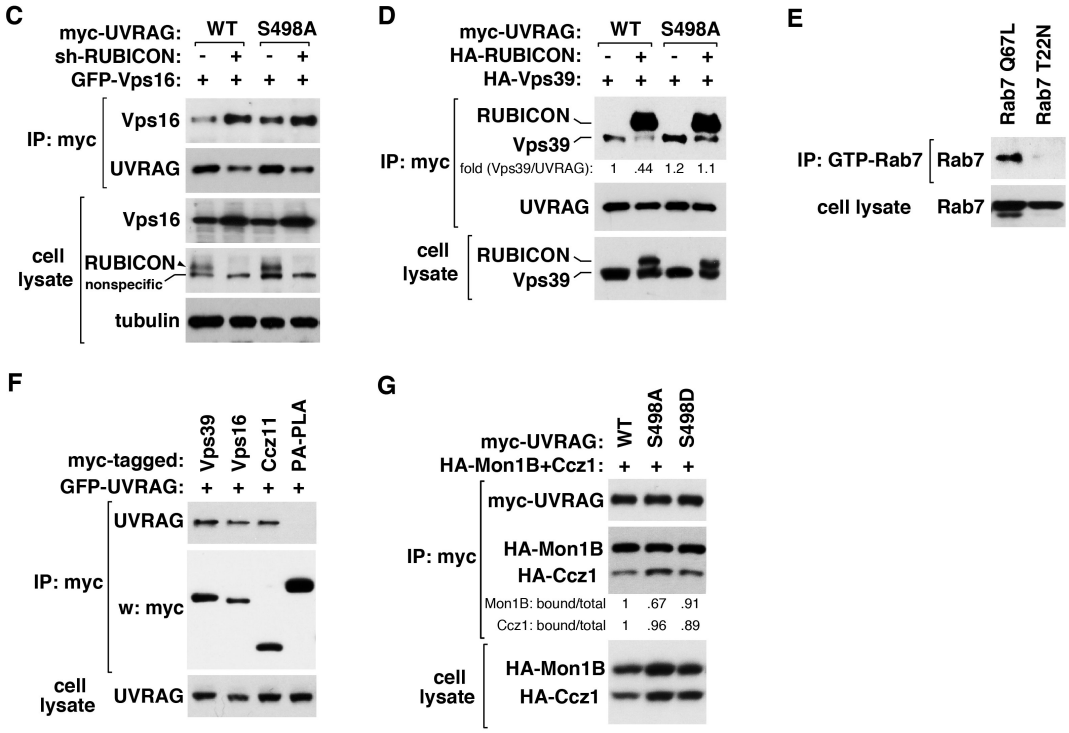

H
myc-UVRAG
WT S498A
IP: HA-Vps34
w: GST-Px
sh-GFP
sh-RUBICON
J
myc-UVRAG
WT S498A
IP: HA-Vps34
w: GST-Px
sh-GFP
sh-RUBICON
I
myc-UVRAG: WT S498A
sh-RUBICON: - + - +
HA-Vps34: + + + +
IP: HA-Vps34
Vps34
cell lysate
RUBICON
myc-UVRAG
myc-Beclin1
K
myc-UVRAG: WT S498A
sh-RUBICON: - + - +
HA-Vps34: + + + +
IP: HA-Vps34
Vps34
cell lysate
RUBICON
myc-UVRAG
L
WT
S498A
relative activity of Vps34
exp1
exp2
- + - + - + - +
sh-RUBICON
M
WT
S498A
fold increase of Vps34 activity by RUBICON knockdown
exp1
exp2
avg.
*

Figure S5. Abolishing the UVRAG Ser498 phosphorylation increases the kinase activity of Vps34. **(A)** The RFP-2xFYVE was transiently expressed together with myc-tagged UVRAG (WT, S498A, or S498D) in HeLa cells where endogenous UVRAG was silenced. 2xFYVE puncta were monitored by fluorescence microscope. Scale bar, 10 μm. (**B**) The RFP-2xFYVE was transiently expressed in HCT116 cells stably expressing UVRAG (empty vector, WT, or S498A). The accumulation of FYVE was analyzed as described in Figure 5A. Scale bar, 5 μm. (**C**) Quantitative analysis of RFP-2xFYVE puncta formation of (B). The error bars represent mean ± SD (n≥55). (*, $p < 0.01$ vs empty vector; $^{\#}$, $p < 0.01$ WT vs S498A). (**D**) 2xFYVE positive area colocalizes with Rab5 and Rab7. HCT116 WT and S498A mutant cells were transiently transduced by RFP-2xFYVE construct together with either GFP-Rab5 or GFP-Rab7. The RFP and GFP positive areas were monitored by fluorescence microscope. Scale bar, 5 μm. (**E**) Preventing UVRAG phosphorylation increases the lipid kinase activity of Vps34. The lipid kinase assay was conducted as described in Figure 5C in the absence or presence of LY294002 (100 μM) for 30 min. (**F**) UVRAG Ser498 phosphorylation is important for the inhibitory effect of RUBICON on UVRAG-induced production of PI3P in cells. HCT116 cells, which were stably transduced by UVRAG constructs, were transiently transduced to express RFP-2xFYVE alone (-) or both RFP-2xFYVE and RUBICON (+). 2xFYVE positive area was monitored by fluorescence microscope. Scale bar, 5 μm. (**G**) RUBICON does not significantly alter the sizes of 2xFYVE positive areas. The error bars represent means ± SD [WT alone (n=121); WT with RUBICON (n=77); S498A alone (n=220); S498A with RUBICON (n=116)]. (**H-K**) The increase of Vps34 kinase activity by RUBICON knockdown is significantly reduced with UVRAG S498A mutation. HEK293T cells that were stably transduced by shRNA were transiently transduced to express the indicated proteins. Two days post-transfection, Vps34 complex was isolated by immunoprecipitation using HA antibody. The *in vitro* kinase assay for Vps34 was conducted as in Figure 5C. (**L, M**) Quantitative analysis of Vps34 kinase activity from H-K. The error bars represent mean ± SD (*, $p<0.05$ between WT and S498A; n=2).

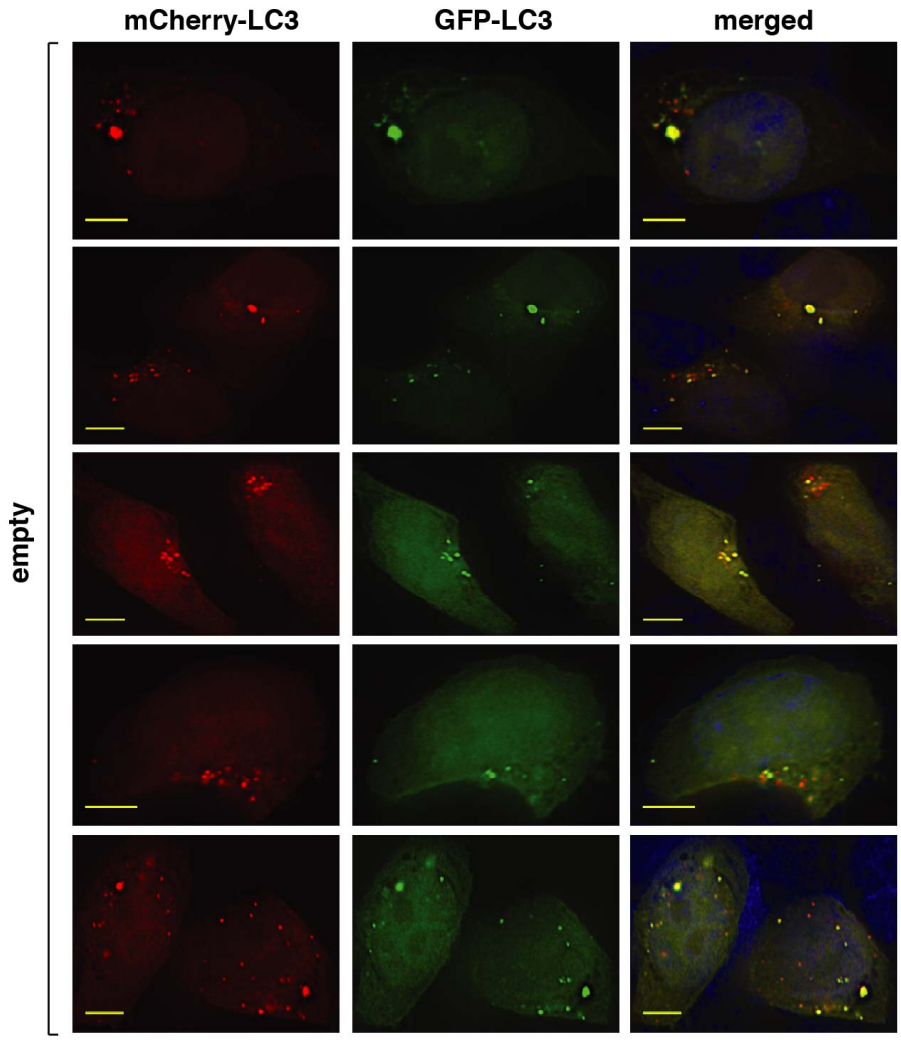
FIGURE S6, related to Figure 6



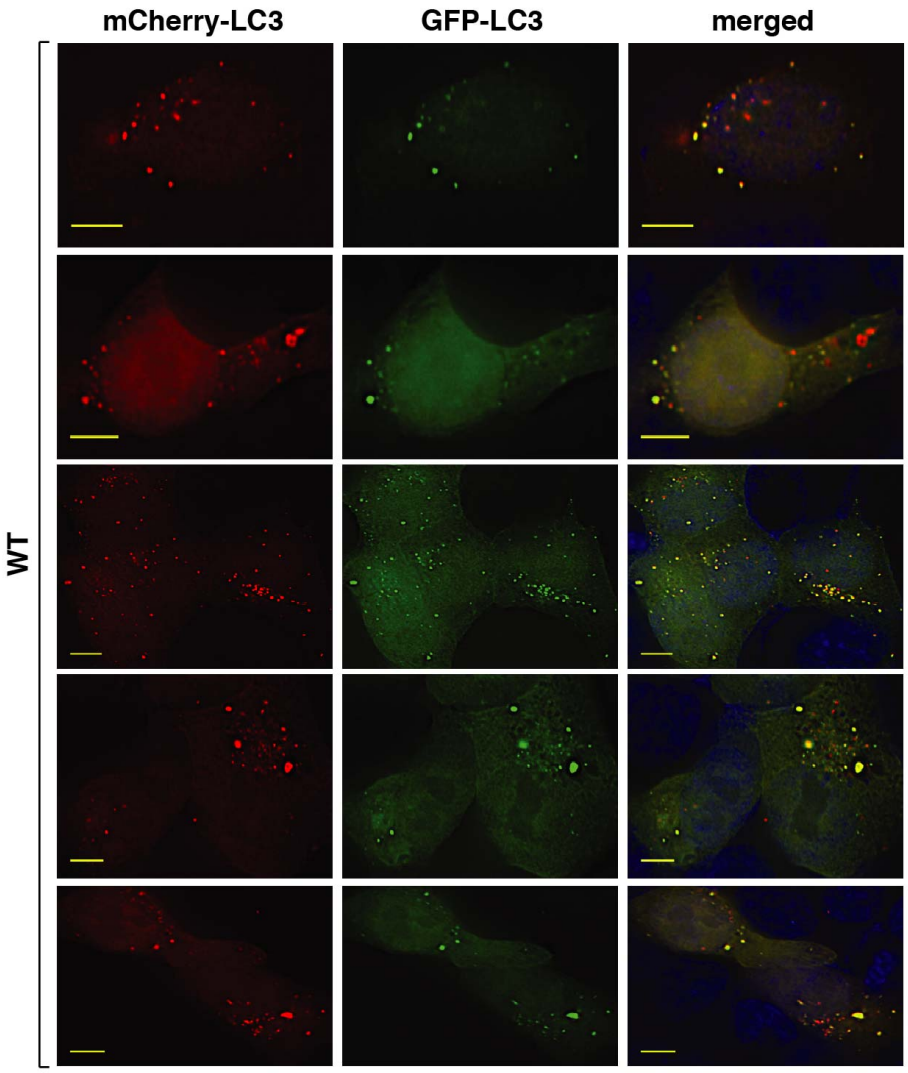

B
+ Torin1
GFP-Vps16
mCherry-UVRAG
merged
UVRAG WT
UVRAG S498A
vps16
UVRAG
Fluorescence Intensity
distance (pixels)


C
myc-UVRAG:
WT
S498A
sh-RUBICON:
- + - +
GFP-Vps16:
+ + + +
IP: myc
Vps16
UVRAG
cell lysate
Vps16
RUBICON
nonspecific
tubulin
D
myc-UVRAG:
WT
S498A
HA-RUBICON:
- + - +
HA-Vps39:
+ + + +
IP: myc
RUBICON
Vps39
fold (Vps39/UVRAG):
1 .44 1.2 1.1
UVRAG
cell lysate
RUBICON
Vps39
E
Rab7 Q67L
Rab7 T22N
IP: GTP-Rab7
Rab7
cell lysate
Rab7
F
myc-tagged:
Vps39
Vps16
Ccz11
PA-PLA
GFP-UVRAG:
+ + + +
IP: myc
UVRAG
w: myc
cell lysate
UVRAG
G
myc-UVRAG:
WT
S498A
S498D
HA-Mon1B+Ccz1:
+ + +
IP: myc
myc-UVRAG
HA-Mon1B
HA-Ccz1
Mon1B: bound/total
1 .67 .91
Ccz1: bound/total
1 .96 .89
cell lysate
HA-Mon1B
HA-Ccz1

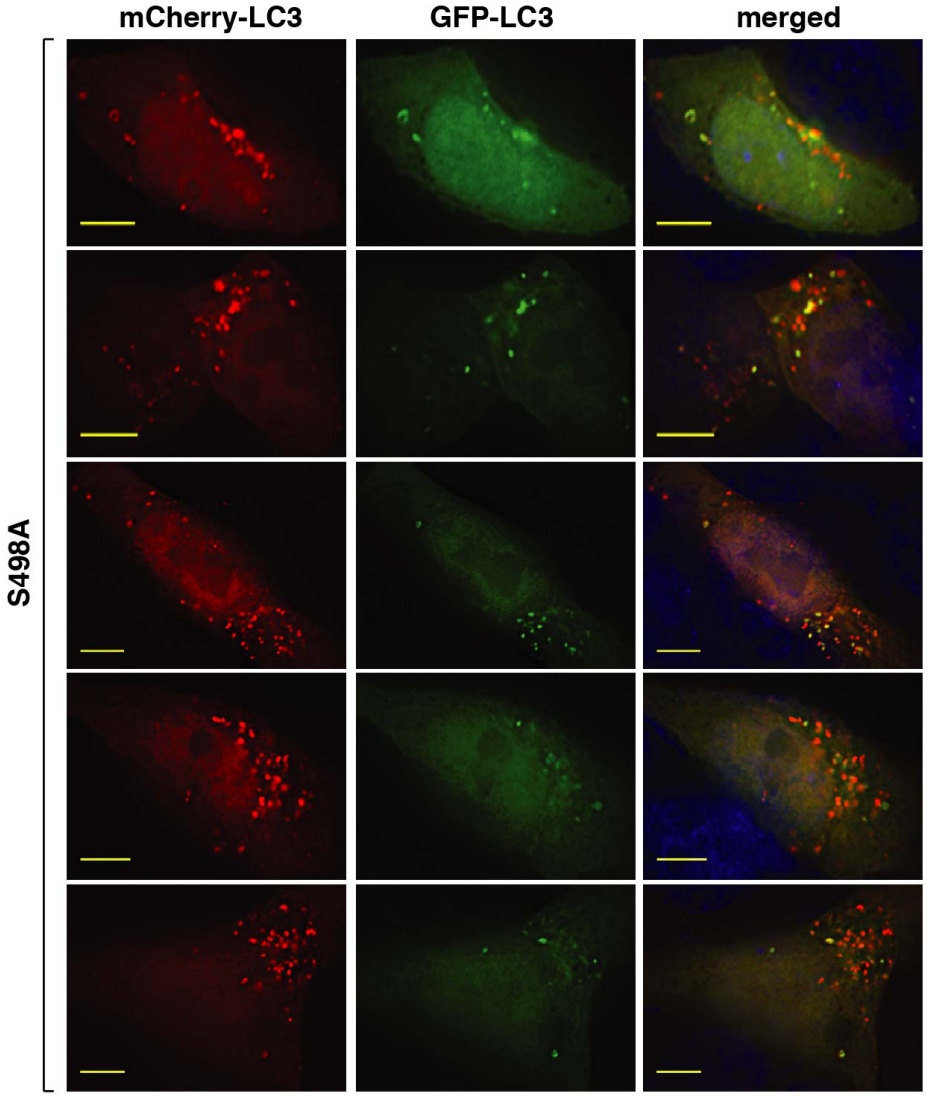
H



I



J



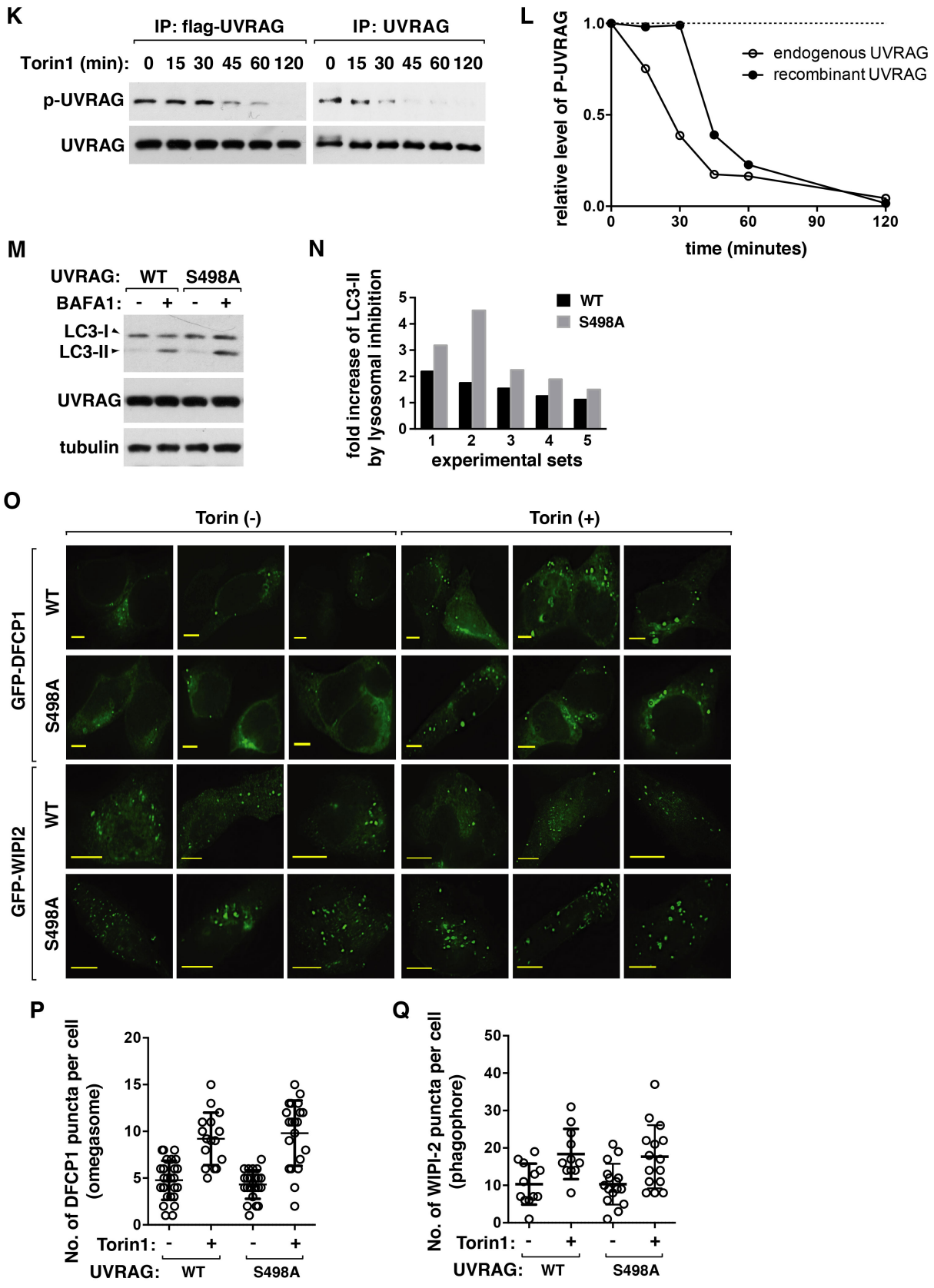
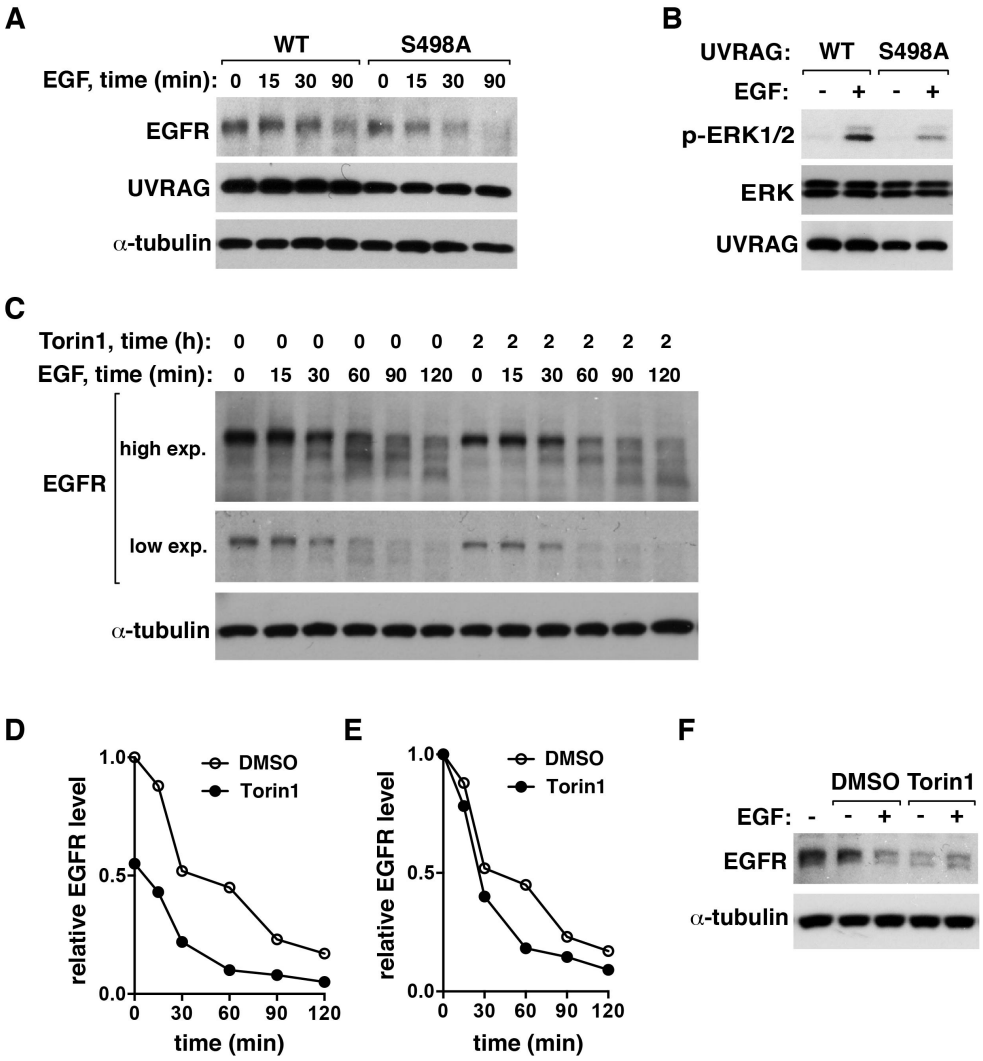

K
IP: flag-UVRAG
IP: UVRAG
Torin1 (min): 0 15 30 45 60 120 0 15 30 45 60 120
p-UVRAG
UVRAG
L
relative level of P-UVRAG
1.0
0.5
0.0
0
30
60
90
120
time (minutes)
endogenous UVRAG
recombinant UVRAG
M
UVRAG: WT S498A
BAFA1: - + - +
LC3-I
LC3-II
UVRAG
tubulin
N
fold increase of LC3-II by lysosomal inhibition
experimental sets
WT
S498A
O
Torin (-)
Torin (+)
GFP-DFCP1
GFP-WIPI2
WT
S498A
P
No. of DFCP1 puncta per cell (omegasome)
Torin1: - + - +
UVRAG: WT S498A
Q
No. of WIPI-2 puncta per cell (phagophore)
Torin1: - + - +
UVRAG: WT S498A

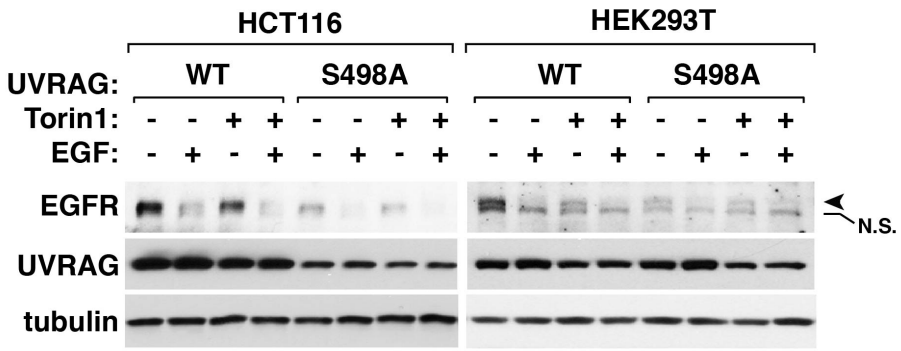
Figure S6. Abolishing the UVRAG Ser498 phosphorylation increases autophagosome maturation. (**A, B**) UVRAG colocalizes with Vps16 more highly in S498A mutant cells compared to WT cells, which is further enhanced by Torin1 treatment. HeLa cells, where endogenous UVRAG was silenced, were transiently transduced to express GFP-Vps16 and mCherry-UVRAG constructs. The transduced cells were analyzed under fluorescence microscope. Intensity line scanes on the right-hand side indicate the degree of colocalization between Vps16 and UVRAG. The line scans were obtained along the yellow lines drawn in the inset boxes of the merged images. Arrows indicate colocalization of UVRAG and Vps16. Scale bar, 5 μm. (**C**) RUBICON knockdown enhances the interaction between UVRAG WT and Vps16 but barely the interaction between UVRAG S498A and Vps16. HEK293T cells stably transduced by shRNA were transiently transduced to express myc-UVRAG and GFP-Vps16 constructs together. The amount of GFP-Vps16 co-immunoprecipitated with myc-UVRAG was analyzed by WB. (**D**) RUBICON overexpression reduces the interaction between UVRAG WT and Vps39 but barely the interaction between UVRAG S498A and Vps39. HEK293T cells, where endogenous UVRAG was silenced, were transiently transduced to express myc-UVRAG and HA-Vps39 constructs together with or without HA-RUBICON. The amounts of HA-RUBICON and HA-Vps39 co-immunoprecipitated with myc-UVRAG were analyzed by WB. (**E**) Validation of the monoclonal antibody that specifically recognizes Rab7-GTP but not Rab7-GDP (Abcam, ab173250). The GFP-Rab7 mutants were transiently expressed in HEK293T cells, and GTP bound Rab7 was immunoprecipitated with the antibody specific to the active state of Rab7 and analyzed by WB using anti-GFP antibody. The active-state mutant of Rab7 (Q67L), but not the inactive-state mutant of Rab7 (T22N), was immunoprecipitated by the antibody. (**F**) Ccz1 interacts with UVRAG with an affinity similar to that of the UVRAG-Vps16/39 interaction. HEK293T cells were transiently transduced to express the indicated myc-tagged constructs together with GFP-UVRAG. The amount of GFP-UVRAG bound to the myc-tagged constructs in myc-immunoprecipitates was analyzed by WB. (**G**) UVRAG Ser498 phosphorylation does not have effects on the interaction between UVRAG and the Mon1/Ccz1 complex. HEK293T cells were transiently transduced to express myc-tagged UVRAG construct together with HA-tagged Mon1B and Ccz1. Two days post-transfection, myc immunoprecipitates were obtained. The amount of HA-Mon1B and HA-Ccz1 in myc immunoprecipitates were analyzed by WB. (**H-J**) UVRAG S498A mutation increases autophagosome maturation. The mCherry-GFP-LC3 construct was transiently expressed in HCT116 cells that were stably transduced by empty vector (H), wild type (I), or S498A (J). The transduced cells were treated with Torin1 (250 nM) for 4 h, and mCherry and GFP puncta were monitored by fluorescence microscope. Scale bar, 5 μm. (**K**) The dephosphorylation of Ser498 of recombinant UVRAG in response to Torin1 is moderately delayed relative to that of endogenous UVRAG. HEK293T cells stably expressing flag-UVRAG were treated with Torin1 (250 nM) for the indicated periods of time. The phosphorylation state of Ser498 of flag-UVRAG in flag immunoprecipitates was analyzed by WB. For comparison, endogenous UVRAG was isolated by immunoprecipitation using anti-UVRAG antibody from HEK293T cells treated with Torin1. (**L**) Quantitative analysis of the relative levels of UVRAG phosphorylation between endogenous and recombinant UVRAG. (**M**) Lysosomal fusion inhibitor BAFA1 increases the level of LC3-II in S498A cells compared

to WT cells. HEK293T cells stably transduced by UVRAG WT or S498A were incubated in DMEM in the presence or absence of Bafilomycin A1 (100 nM) for 4 h. (**N**) Quantitative analysis of the fold increase of LC3-II level induced by the treatment with lysosomal inhibitors in Torin1-treated WT and S498A mutant cells. (**O**) Ser498 phosphorylation does not have effects on omegasome and phagophore formation. GFP-DFCP1 was transiently expressed in HCT116 WT and S498A mutant cells, whereas GFP-WIPI2 was transiently expressed with myc-tagged UVRAG (WT or S498A) in HeLa cells, where endogenous UVRAG is silenced. Cells were treated with Torin1 (250 nM) for 4 h, and GFP-DFCP1 and GFP-WIPI2 were monitored by fluorescence microscope. (**P, Q**) Quantitative analysis of DFCP1 puncta (P) and WIPI2 puncta from (Q). Mean value and SD are shown as a horizontal bar. Scale bars are 5 μm and 10 μm for DFCP1- and WIPI2-stained images, respectively.

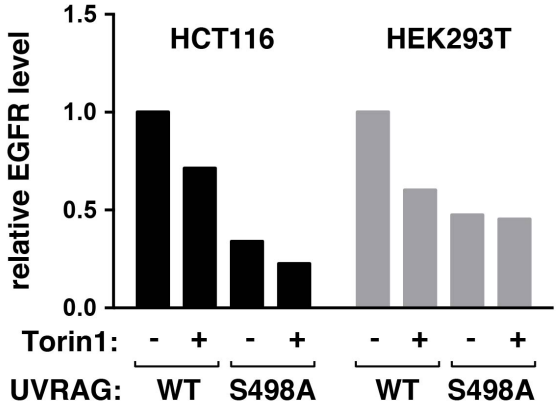
FIGURE S7, related to Figure 7



G


HCT116
HEK293T
UVRAG:
WT
S498A
WT
S498A
Torin1:
EGF:
EGFR
N.S.
UVRAG
tubulin

H


relative EGFR level
1.5
1.0
0.5
0.0
HCT116
HEK293T
Torin1:
UVRAG:
WT
S498A
WT
S498A

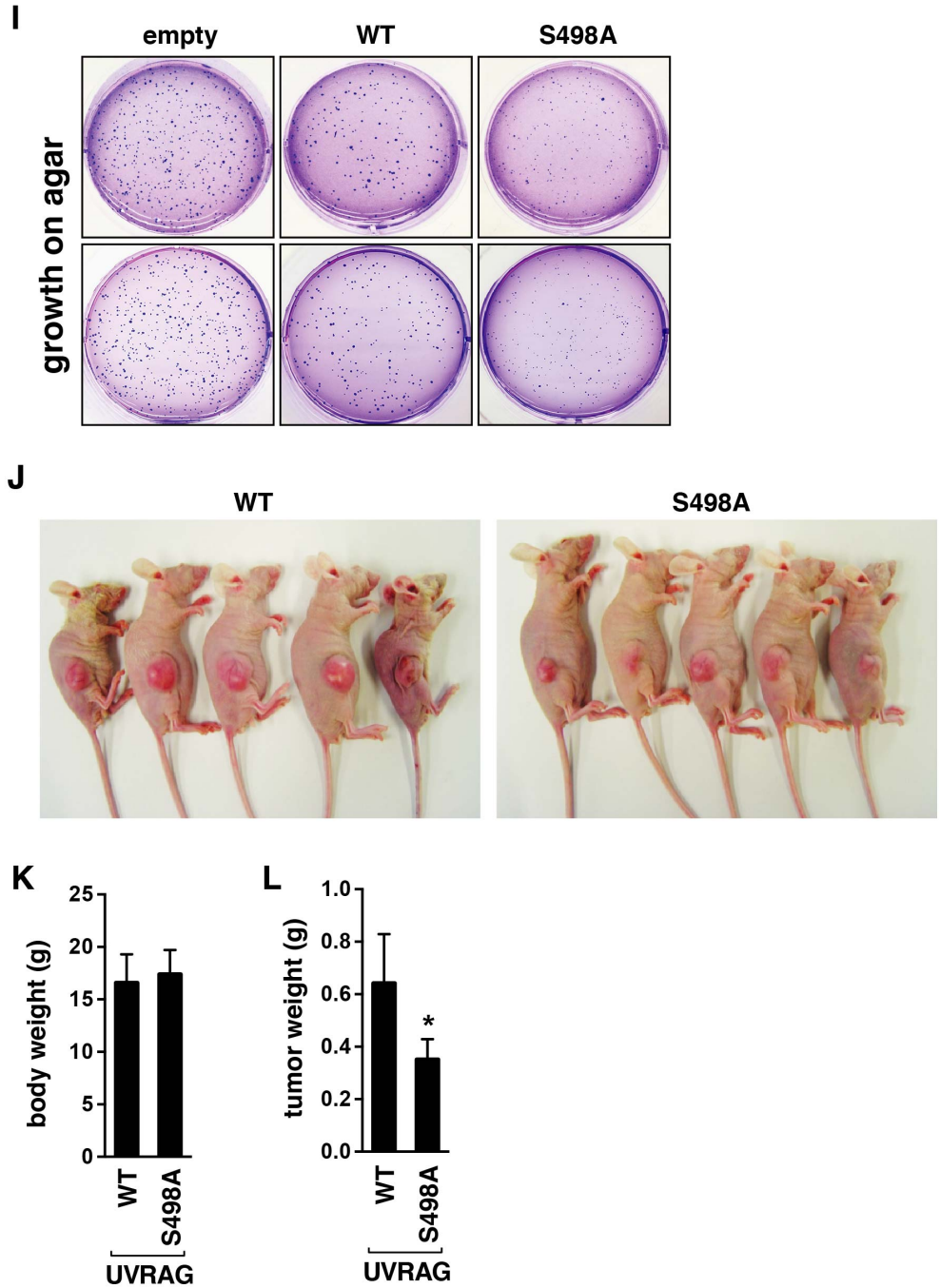

I
empty
WT
S498A
growth on agar
J
WT
S498A
K
body weight (g)
25
20
15
10
5
0
WT
S498A
UVRAG
L
tumor weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
*
WT
S498A
UVRAG

Figure S7. Prevention of UVRAG Ser498 phosphorylation enhances endosome-lysosomal degradation of EGFR, reduces EGFR signaling, and suppresses cancer cell and tumor growth. (**A**) Preventing UVRAG Ser498 phosphorylation increases the lysosomal degradation of EGFR. HCT116 cells that stably express flag-UVRAG (WT or S498A) were treated with EGF (100 ng/ml) for the indicated periods of time. The level of EGFR was monitored by WB. (**B**) Preventing UVRAG Ser498 phosphorylation suppresses EGF-stimulated ERK1/2 phosphorylation. HCT116 cells that stably expressing flag-UVRAG WT or S498A were starved overnight and treated with EGF (20 ng/ml) for 10 min. (**C**) Torin1 facilitates the degradation of EGFR. HEK293T cells were starved in serum-free media overnight and treated with vehicle (DMSO) or Torin 1 (250 nM) for 2 h, then stimulated with EGF (100 ng/ml). (**D and E**) Quantitative analysis of EGFR degradation in Figure S7C. The level of EGFR was presented relative to that at time zero for DMSO (D) or relative to those at time zero for DMSO and Torin1, respectively (E). (**F**) Torin1 reduces the basal level of EGFR. HEK293T cells were serum starved overnight, incubated DMSO or Torin (250 nM) for 4 h, and followed by treatment of EGF (10 ng/ml). (**G**) Torin1 marginally reduces the basal level of EGFR in S498A mutant cells relative to WT cells. The cells were treated with Torin1 and EGF as described in (F). (**H**) Quantitative analysis of the levels of EGFR of (G). (**I**) UVRAG S498A mutation significantly inhibited cancer cell colony formation. HCT116 cells ($1x10^4$/6 well plate), prepared as described in Figure 7D, were seeded and incubated in soft agar for 2 weeks. The colony was stained with 0.005% crystal violet as described in Methods. (**J-L**) Abolishing UVRAG phosphorylation suppressed tumor growth *in vivo*. HCT116 cells stably expressing flag-UVRAG WT or S498A were subcutaneously injected into flank of Balb/c nu/nu mice (n=5). Tumor-bearing mice were pictured (J) and weighed (K) before they were sacrificed. Tumors were removed from the mice and weighed (L). The error bars represent mean ± SD (n = 5). (*, $p < 0.01$, WT vs S498A).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials

Anti-S6K1 (9202), anti-phospho-S6K1 Thr389 (9205), anti-Akt (9272), anti-phospho-Akt Thr308 (9275), anti-4EBP1 (9644), anti-phospho-4EBP1 Thr37/46 (9459), anti-phospho ERK Thr202/Tyr204 (9101), anti-ERK (9102), anti-Vps34 (3811), and anti-mTOR antibodies (2972) were from Cell Signaling Technology (Danvers, MA). Anti-UVRAG (82115), anti-Rubicon (99955), anti-Beclin1 (11427), anti-GFP (9996), anti-EGFR (03), anti-TSC1 (12082), anti-TSC2 (893), anti-Atg14L (164767), and anti-tubulin (12462) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-UVRAG (M160-3) and anti-RUBICON (PD027) were from MBL (Woburn, MA). Antibodies against myc 9E10 (OP10), HA.11 (MMS-101R), flag (F3165), LC3 (5F10), and Vps34 (Z-R015) were purchased from EMD biomedicals (Bedford, MA), Covance (Princeton, NJ), Sigma-Aldrich (St Louis, MO), NanoTools (Teningen, Germany), and Echelon Biosciences (Salt Lake City, UT), respectively. Anti-GST (27457701) antibody was from GE Healthcare Life Sciences. Rab7 activation assay kit (ab173250) was purchased from Abcam (Cambridge, England). Anti-raptor and anti-rictor antibodies were described in our previous report (Vander Haar et al., 2007). Anti-phospho-UVRAG S498 antibody was generated in rabbit using a UVRAG peptide GFSGGIP**s***PDKGHRK, where the asterisk-labeled serine was modified with phosphate group, conjugated to KLH by AbFrontier (Seoul, South Korea). DMEM (11995), HBSS (24020), Opti-MEM (31985), EGF (13247-051), Lipofectamine 2000 (11668-019), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (M-6494) were from Life Technologies (Carlsbad, CA). X-tremeGene 9 DNA transfection reagent (06365787001)

and Fugene 6 (E2692) were purchased from Roche Diagnostics (Indianapolis, IN) and Promega (Madison, WI), respectively. Torin1 (4247), α-Phosphatidylinositol (840042P), PI3P-Grip (G-0302), and Sea plaque agarose (50101) were from R&D (Minneapolis, MN), Avanti Polar Lipids (Alabaster, AL), Echelon Biosciences (Salt Lake City, UT), and Lonza (Rockland, ME), respectively. All other reagents were purchased from Sigma unless indicated otherwise.

Mass Spectrometry Identification of UVRAG Phosphorylation Sites

Tryptic digests of peptides were dried and resuspended in load solvent (98:2:0.01, water: acetonitrile:formic acid). About 1-1.5 ug of the digested samples were run on a Velos Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA) as described previously (Lin-Moshier et al., 2013). The mass spectrometer raw data (Proteowizard files) were converted to mzXML using MSconvert software 9 http://proteowizard.sourceforge.net/), then converted to DTA files with an in-house script (http://github.com/jmchilton/tint/). All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, version 27, rev. 12). The digestion enzyme trypsin fragment ion mass tolerance was 0.80 Da and precursor tolerance was 1.00 Da. Oxidation of methionine and phosphorylation of serine and threonine were specified in Sequest as variable modifications. Scaffold (version Scaffold 4.2.1., Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identification. Peptide identifications were accepted if they could be established at greater than 95% probability by the Peptide Prophet algorithm (Keller et al., 2002).

Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Peptides of phosphorylated candidates were manually verified for accuracy.

Cell Culture, Transfection and Treatment

HEK293T, MEF, HeLa, and HCT116 cells were cultured in DMEM containing 10% fetal bovine serum and penicillin/streptomycin at 37^{o}C in 5% CO_2. For transient expression, cells were transfected with recombinant DNAs using Fugene 6, X-tremeGene 9 DNA transfection reagent or Lipofectamine 2000 following the manufacturer's protocols. After two days, cells were harvested or used for immunoprecipitation or microscopic analysis. For leucine treatment, cells were incubated in leucine-deprived medium for 40 min, then supplemented with leucine (104 μg/ml) for 30 min. For the treatments with Torin1, rapamycin and Akt inhibitor VIII, the final concentrations were 250 nM, 100 nM and 10 μM, respectively, unless stated otherwise. For nutrient starvation, cells were incubated in HBSS without serum for 2 h unless stated otherwise.

Mutagenesis

Mutagenesis was performed to generate UVRAG mutant constructs using a site-directed mutagenesis kit (Stratagene, LaJolla, CA). The sequences of primers that were used for mutagenesis is: UVRAG (S498A: TCTGGGGGGATCCCTGCACCAGACAAAGGACAT; S498D: TCTGGGGGGATCCCTGACCCAGACAAAGGACATC).

Immunoprecipitation and Western Blotting

For coimmunoprecipitation experiments, whole-cell extracts were prepared in 0.3% Chaps-containing lysis buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1.5 mM Na_3VO_4, 10 mM β-glycerophosphate, 0.3% Chaps, and EDTA-free protease inhibitors) as described in a previous report (Kim et al., 2002), and immunoprecipitated with anti-UVRAG, anti-RUBICON, anti-Vps34, anti-Atg14L, anti-mTOR, anti-raptor, anti-rictor, anti-HA, or anti-Myc antibody. Precipitated proteins were washed four times with 0.3% Chaps lysis buffer, loaded onto Tris–glycine gels (Invitrogen), transferred for 4 hrs onto immunoblot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA), incubated with primary and secondary antibodies, and detected with ECL western blotting detection reagents (Advansta).

Lentiviral Preparation and Stable Cell Generation

To prepare lentivirus for knockdown experiment, HEK293T cells were transduced by pLKO.1 harboring an shRNA target sequence and lentiviral packaging vectors pHR'8.2ΔR and pCMV-VSV-G using Lifopectamine 2000 (Life Technologies). The target sequences are 5'-GCCCTTGGTTATACTGCAC-3' for human UVRAG 3'-UTR and 5'-CGAGAGCAGCAGTTCCAAT-3' for human RUBICON. To prepare lentivirus for UVRAG expression, HEK293T cells were transduced by CSII-EF-MCS empty vector control or vector encoding flag-UVRAG WT or S498A with packaging vectors ΔNRF-HIV-1-gag-pol and pCMV-VSV-G using Lifopectamine 2000 (Life Technologies). Forty-eight hours post transfection, virus-containing medium was collected. Target cells (HEK293T, HeLa, and HCT116 cells) were incubated with the collected viral supernatant for 4 h at 37°C, then cells were refreshed and further incubated with

medium containing viral supernatant overnight at 37°C in the presence of polybrene. Stably transduced cells were selected in the presence of puromycin or zeocin.

Preparation of Recombinant Proteins

The human UVRAG full length (WT or S498A) and fragments (1-170, 1-300, 271-699, 151-300, 301-500, and 401-699) were cloned in pGEX-6P-2 (GE Healthcare) and expressed as GST-tagged forms in ArcticExpress bacteria (Stratagene) by induction with 1 mM IPTG for 20 h at 12.5°C. The recombinant proteins were purified using glutathione-sepharose 4B beads followed by digestion with PreScission protease (GE Healthcare, 27-0843-01) that removes GST from the fusion constructs.

In Vitro Assay of UVRAG Phosphorylation by mTOR

mTOR immuno complex was isolated using anti-mTOR, raptor, or rictor antibodies from HEK293T or HeLa cells. Alternatively, myc-tagged mTOR WT or KD was isolated from HEK293T expressing the mTOR construct. We also used a purified active form of mTOR containing residues 1362-2549 (Millipore, Bedford, MA). UVRAG fragments (1-170, 1-300, 271-699, 151-300, 301-500, or 401-699) and its full length WT and S498A were purified from ArcticExpress bacteria (Stratagene) using GSH resin affinity purification. In vitro kinase reaction was conducted at 30°C in a buffer (50 mM HEPES, pH 7.5, 1 mM EGTA, 0.01% Tween 20, 10 mM $MnCl_2$, 2.5 mM and DTT) containing 1 μg of the UVRAG substrate in the presence or absence of 100 μM ATP and/or the trace amount of [γ-^{32}P]ATP. The reaction was terminated by addition of SDS-sample loading buffer. The reaction mixture was loaded on SDS-PAGE, and the phosphorylation state

was analyzed by autoradiography or WB using anti-phospho-UVRAG antibody (pSer498).

Cell Image Analysis

For monitoring the PI3P accumulation in cells, RFP-tagged 2xFYVE was transiently expressed in WT or mutant UVRAG-transduced HCT116 cells or in WT or mutant UVRAG-reconstituted HeLa cells. RFP-2xFYVE puncta formation was monitored by fluorescence microscope. For autophagosome maturation assay, mCherry-GFP-LC3 was transiently expressed in HCT116 cells stably transduced by empty vector or stably expressing WT or S498A UVRAG for 2 days. Cells were treated with vehicle (DMSO) or Torin1 (250 nM) for 4 h. For omegasome formation analysis, GFP-DFCP1 was expressed in HCT116 cells stably expressing UVRAG WT or S498A. For phagophore formation analysis, GFP-WIPI2 was expressed with myc-UVRAG (WT or S498A) in UVRAG-depleted HeLa cells. To analyze the colocalization of 2xFYVE with endosomal compartments, RFP-tagged 2xFYVE was transiently expressed with either GFP-tagged Rab5 or Rab7 in HCT116 WT and S498A cells. Two days post-transfection, cells were fixed with 4% paraformaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI) for the nuclei. Stained cells were placed in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized under a Deltavision Personal DV fluorescence microscope (Applied Precision, Issaquah, WA).

In Vitro Assay of Vps34 Kinase Activity

In vitro Vps34 activity assay was performed as described previously (Farkas et al., 2011). Briefly, Vps34 immunoprecipitates were isolated using either anti-Vps34 (Echelon Biosciences) or anti-HA antibody in lysis buffer (50 mM Tris pH 7.4, 7.5% glycerol, 150 mM NaCl, 1 mM EDTA, and protease inhibitors). The isolated immunoprecipitates were washed three times with the lysis buffer and once with substrate buffer (75 mM Tris, pH7.5, 125 mM NaCl, and 12.5 mM $MnCl_2$). The beads were incubated in substrate buffer containing phosphatidylinositol (250 μg/ml) on ice for 10 min and at room temperature for 5 min. The Vps34 kinase reaction was initiated by addition of ATP (10 μM) and processed at room temperature for 30 min. The reaction mixture was spotted on nitrocellulose membrane. The membrane was incubated with 1% fat milk in PBS for 2 h, and further incubated with 0.5 μg/ml of GST-tagged p40 phox domain-containing polypeptide in PBS containing 3% BSA and 0.1% Tween 20 for 2 h. The amount of PI3P on the nitrocellulose membrane was assessed by WB of the GST-tagged p40 phox domain polypeptide using anti-GST antibody.

Analysis of Rab7 Active State

The active state of Rab7 was assayed according to the manufacturer protocol (Abcam, ab173250). Briefly, myc-tagged UVRAG (WT or S498A) was expressed with GFP-tagged Rab7 in UVRAG-depleted HEK293T cells. Two days post-transfection, cells were lysed with the 1x assay/lysis buffer provided by the manufacturer. One microliter of anti-active Rab7 monoclonal antibody was added to the cell lysate with Prointe A/G agarose to immunoprecipitate the active state of Rab7 for 1 h at 4°C with gentle

agitation. The active-Rab7 immunoprecipitates were washed three times with 1x assay/lysis buffer. The precipitated proteins were analyzed by WB.

Autophagy Flux Assay

HEK293T cells stably expressing flag-UVRAG WT or S498A were treated with Torin1 (250 nM) for 4 h in the presence or absence of lysosomal inhibitors (Bafilomycin A1 or E-64/Pepstatin A). The final concentrations of Bafilomycin A1 and E-64/Pepstatin A were 100 nM and 10 μg/mL each, respectively. Cells were lysed with lysis buffer containing 40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1.5 mM Na_3VO_4, 10 mM β-glycerophosphate, 1% Triton X-100, and EDTA-free protease inhibitors. The amounts of LC3-I and LC3-II were analyzed by WB.

Cell Proliferation Assay

Cell proliferation was assayed by counting cell number or monitoring MTT-stained viable cells. For cell number counting, $2x10^5$ of HCT116 cells were seed onto 6 well plates and cultured for the indicated periods of time. Viable cells were counted using a hematocytometer. For MTT assay, $1x10^4$ cells of HCT116 cells were split onto 96 well plates and cultured for the indicated periods of time. At the indicated days, 10 μl of MTT (1 μg/well) was added into each well. Cells were further incubated for 2 h. DMSO was added to dissolve MTT formazan crystals. MTT-formazan crystals were monitored by measuring the absorbance at the wavelength of 595 nm using a spectrophotometer.

Colony Formation Assay

HCT116 cells ($1x10^4$ cells) stably transduced by empty vector or stably expressing flag-UVRAG WT or S498A were suspended in 0.48% agarose in complete growth medium, and plated onto 6 well plates over a bottom layer of 0.8% agarose in complete growth medium. One milliliter of complete growth media was added into each well and refreshed every 4-5 days. After 2 weeks, media was removed, and colonies were stained with 0.005% crystal violet in PBS containing 3.7% formaldehyde for 1 h. After the crystal violet staining solution was removed, colonies were analyzed using image J (Size [pixle^2]: 100-infinity).

Xenograft Analysis of Tumor Formation

HCT116 cells (1×10^7) stably expressing UVRAG (wild type or S498A) were suspended in 0.2 ml culture medium and then injected subcutaneously into the right flank of athymic nude male mice (BALB/c nu/nu, 4 weeks old). Three weeks after the injection, tumor-bearing mice were pictured, weighed, and sacrificed. For measurement of tumor volume, tumors were removed from mice and weighed. Tumor volume was evaluated by measurement of the length and width of the tumor mass in millimeters (mm) using electronic vernier calipers. Tumor volume (mm^3) was calculated using the formula V = (length × width × height)/2.

Statistical analysis

Western blot band intensities were quantified by Image J software. Cellular puncta and their colocalization were quantitatively analyzed by counting the number of puncta above an intensity threshold using Image J software. The quantitfied values were

summarized by the means along with either standard errors or standard deviations. Statistical significance was assessed by the Student's *t*-test using Prism 6.0d (GraphPad Software, Inc., La Jolla, CA).

SUPPLEMENTAL REFERENCES

Farkas, T., Daugaard, M., and Jaattela, M. (2011). Identification of small molecule inhibitors of phosphatidylinositol 3-kinase and autophagy. J Biol Chem *286*, 38904-38912.

Jung, C.H., Jun, C.B., Ro, S.H., Kim, Y.M., Otto, N.M., Cao, J., Kundu, M., and Kim, D.H. (2009). ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell *20*, 1992-2003.

Kang, S.A., Pacold, M.E., Cervantes, C.L., Lim, D., Lou, H.J., Ottina, K., Gray, N.S., Turk, B.E., Yaffe, M.B., and Sabatini, D.M. (2013). mTORC1 phosphorylation sites encode their sensitivity to starvation and rapamycin. Science *341*, 1236566.

Keller, A., Nesvizhskii, A.I., Kolker, E., and Aebersold, R. (2002). Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem *74*, 5383-5392.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163-175.

Liang, C., Lee, J.S., Inn, K.S., Gack, M.U., Li, Q., Roberts, E.A., Vergne, I., Deretic, V., Feng, P., Akazawa, C.*, et al.* (2008). Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. Nat Cell Biol *10*, 776-787.

Lin-Moshier, Y., Sebastian, P.J., Higgins, L., Sampson, N.D., Hewitt, J.E., and Marchant, J.S. (2013). Re-evaluation of the role of calcium homeostasis endoplasmic reticulum protein (CHERP) in cellular calcium signaling. J Biol Chem *288*, 355-367.

Nesvizhskii, A.I., Keller, A., Kolker, E., and Aebersold, R. (2003). A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem *75*, 4646-4658.

Sun, Q., Westphal, W., Wong, K.N., Tan, I., and Zhong, Q. (2010). Rubicon controls endosome maturation as a Rab7 effector. Proc Natl Acad Sci U S A *107*, 19338-19343.

Vander Haar, E., Lee, S.I., Bandhakavi, S., Griffin, T.J., and Kim, D.H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat Cell Biol *9*, 316-323.

Woo, S.Y., Kim, D.H., Jun, C.B., Kim, Y.M., Haar, E.V., Lee, S.I., Hegg, J.W., Bandhakavi, S., and Griffin, T.J. (2007). PRR5, a novel component of mTOR complex 2, regulates platelet-derived growth factor receptor beta expression and signaling. J Biol Chem *282*, 25604-25612.